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Functional analysis of seven genes encoding eight translation initiation factor 4E (eIF4E) isoforms in *Drosophila*

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Abstract

The *Drosophila* genome-sequencing project has revealed a total of seven genes encoding eight eukaryotic initiation factor 4E (eIF4E) isoforms. Four of them (*eIF4E-1,2*, *eIF4E-3*, *eIF4E-4* and *eIF4E-5*) share exon/intron structure in their carboxy-terminal part and form a cluster in the genome. All eIF4E isoforms bind to the cap (m⁷GpppN) structure. All of them, except eIF4E-6 and eIF4E-8 were able to interact with *Drosophila* eIF4G or eIF4E-binding protein (4E-BP). eIF4E-1, eIF4E-2, eIF4E-3, eIF4E-4 and eIF4E-7 rescued a yeast eIF4E-deficient mutant in vivo. Only eIF4E-1 mRNAs and, at a significantly lower level, eIF4E3 and eIF4E-8 are expressed in embryos and throughout the life cycle of the fly. The transcripts of the remaining isoforms were detected from the third instar larvae onwards. This indicates the cap-binding activity relies mostly on eIF4E-1 during embryogenesis. This agrees with the proteomic analysis of the eIF4F complex purified from embryos and with the rescue of *l(3)67Af*, an embryonic lethal mutant for the *eIF4E-1,2* gene, by transgenic expression of eIF4E-1. Overexpression of eIF4E-1 in wild-type embryos and eye imaginal discs results in phenotypic defects in a dose-dependent manner.

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1. Introduction

Translation of eukaryotic mRNAs involves recognition of the cap structure m⁷GpppN present at the 5' end of mRNAs by eukaryotic initiation factor 4E (eIF4E). In association with eIF4G, eIF4E forms the eIF4F complex that allows binding of 40S ribosomal subunits probably through association with eIF3. Together with the helicase protein complex formed by eIF4A and eIF4B, eIF4F unwinds the secondary structure at the 5' untranslated

region (UTR) of most mRNAs. This event promotes the landing of 40S ribosomal subunits and the subsequent search of the initiator codon (Gingras et al., 1999). Due to its central role in cap-dependent translation, regulation of eIF4E activity is critical to normal cell growth. Overexpression of eIF4E in cell cycle-sensitized or proto-oncogenic cells results in overgrowth and malignant transformation. eIF4E expression is also significantly increased in many cancers (de Benedetti and Graff, 2004; Dua et al., 2001; Rosenwald, 2004; Sonenberg and Gingras, 1998). The recently discovered structural features of eIF4E shed light on the biological properties of this factor. The three-dimensional structure of yeast, mouse and human eIF4E in complex with different cap analogs has been resolved (Gross et al., 2003; Marcotrigiano et al., 1997;

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Matsuo et al., 1997; Tomoo et al., 2002, 2003). The amino-terminal part of eIF4E is unstructured, highly flexible and is completely separated from the functional moiety of the protein (Gross et al., 2003; Matsuo et al., 1997; Tomoo et al., 2002, 2003). In addition, the amino-terminal moiety is highly divergent across the phyla, is dispensable for cap recognition, for binding to eIF4G and 4E-BP, for stimulation of cap-dependent translation, and for in vivo functionality of the protein (Gross et al., 2003; Marcotrigiano et al., 1997; Robalino et al., 2004; Vasilescu et al., 1996). Conversely, the sequence and three-dimensional structure of eIF4E carboxy-terminal part is highly conserved and contains all the residues important for its functionality. Its shape resembles a baseball glove in whose cavity the guanine ring of the cap is stacked by π – π interactions between the lateral chains of amino acids W56 and W102 (numbers refer to mouse eIF4E). E103 and W102 as well as the interaction between W166 and the methyl group of the cap structure further stabilize this interaction. Positive charges of R112, R157 and R162 interact with the negatively charged phosphate residues of the cap (Gross et al., 2003; Marcotrigiano et al., 1997; Matsuo et al., 1997; Tomoo et al., 2002, 2003). Additional contacts between the second nucleotide of m⁷GpppA and the carboxy-terminal part (residues 204–211) of human eIF4E have been recently identified (Tomoo et al., 2002, 2003). Moreover, eIF4E interacts with eIF4G and with the negative regulators eIF4E-binding proteins (4E-BPs) through several conserved residues present in its carboxy-terminal moiety, located in the convex dorsal surface of the protein (Gross et al., 2003; Marcotrigiano et al., 1999; Matsuo et al., 1997; Ptushkina et al., 1998). Phosphorylation of mammalian eIF4E S209 is very well documented to occur in response to extracellular signals (Flynn and Proud, 1995; Joshi et al., 1995; Pyronnet et al., 1999; Waskiewicz et al., 1999). Although the physiological importance of S209 in the activity of mammalian eIF4E has been recently challenged (McKendrick et al., 2001; Scheper et al., 2002), the phosphorylation of an equivalent to S209 in *Drosophila* eIF4E-1 (S251) has been proven to be critical for growth (Lachance et al., 2002).

The genome-wide sequencing projects have started to reveal the presence of several genes encoding eIF4E in different organisms, but the physiological relevance of this is still not known. Three different eIF4E proteins have been studied in mammals, eIF4E-1 (Rychlik et al., 1987), 4EHP (Rom et al., 1998) and eIF4E-3 (Joshi et al., 2004), and many others genes can be identified from the human raw sequence (A. Andrei and R. Rivera-Pomar, unpublished); three isoforms are known in plants termed eIF4E, eIF(iso)4E (Browning, 1996) and novel cap-binding protein (nCBP) (Ruud et al., 1998); at least five isoforms of eIF4E exist in *C. elegans* (Keiper et al., 2000), two in Zebrafish (Fahrenkrug et al., 1999; Robalino et al., 2004), two in *Xenopus* (Wakiyama et al., 2001), and two in *S. pombe* (Ptushkina et al., 1996, 2001). In *S. cerevisiae* only a single

essential gene encoding for eIF4E has been identified (Altmann et al., 1987).

In *Drosophila*, the translational control of maternal genes plays a key role during embryogenesis (Johnstone and Lasko, 2001). On the other hand, eIF4E is an important target of regulatory factors such as BICOID to regulate the cap-dependent translation of *caudal* mRNA (Niessing et al., 1999, 2002), and CUP for the regulation of *oskar* mRNA translation (Nakamura et al., 2004; Wilhelm et al., 2004). In order to understand the role of the different eIF4E isoforms in *Drosophila* embryo, we studied the expression and biochemical properties of all eIF4E genes from *Drosophila*. We found that *Drosophila* possesses seven genes encoding eight eIF4E polypeptides, and we established by genetic and biochemical means that during embryogenesis eIF4E-1 plays an essential role and that the cap-binding activity relies only on this isoform.

2. Results

2.1. Seven genes encode eight eIF4E isoforms in *Drosophila*

Early studies performed with embryos characterized the polypeptide and the cDNA encoding for *Drosophila* eIF4E-1 (Hernández and Sierra, 1995; Maroto and Sierra, 1989). It was later reported that a single gene, *eIF4E-1,2*, encodes for two isoforms, eIF4E-1 and eIF4E-2, as a result of alternative splicing (see Fig. 2) (Hernández et al., 1997; Lavoie et al., 1996). The completion of the sequencing project of *Drosophila* genome (Adams et al., 2000) led to the annotation of five new *eIF4E* genes (Lasko, 2000) and a seventh one was annotated by the Ensembl Project (Sanger Institute) (Birney, 2004), encoding a total of eight eIF4E isoforms. An important discrepancy, however, was noticed for the cDNA of CG32859 gene (encoding for eIF4E-7) reported by the above sources. Although the predicted sequence of six detected *Drosophila* eIF4E cDNAs coincided in both analyses, there has been no experimental validation for the sequences and for the functionality of the encoded polypeptides. We cloned the eight *Drosophila* eIF4E cDNAs from ESTs or from a cDNA library and sequenced both strands, confirming the sequences previously reported by the Ensemble Project (Birney, 2004). The annotated *eIF4E* genes, their chromosomal location, and the predicted length and molecular mass of the polypeptides are listed in Table 1. An alignment of the *Drosophila* polypeptides together with the sequence of mouse (Jaramillo et al., 1991) and yeast eIF4Es (Altmann et al., 1987) is shown in Fig. 1a. All *Drosophila* eIF4Es, in particular eIF4E-7, have long amino-terminal regions (from 31 to 249 amino acids) as compared to eIF4E from other species. This makes eIF4E-7 the largest eIF4E so far known in any organism. The amino-terminal region is highly divergent in all the proteins compared, as well as in eIF4Es from other species. In contrast, all polypeptides share high

Table 1
Drosophila melanogaster eIF4E isoforms

Gene	Chromosome localization	Isoform encoded	Length (aa)	Predicted M. W. (Da)
<i>eIF4E1,2</i> ^a	67A5 (3L)	eIF4E-1	259	29.223
<i>eIF4E1,2</i> ^a	67A5 (3L)	eIF4E-2	248	27.829
CG8023	66C1(3L)	eIF4E-3	244	28.494
CG10124	65C4(3L)	eIF4E-4	229	26.374
CG8277	66A18 (3L)	eIF4E-5	232	26.938
CG1442	98F13 (3R)	eIF4E-6	173	20.146
CG32859	1E3 (X)	eIF4E-7	429	48.713
CG33100	95E1 (3R)	eIF4E-8	223	25.775

^a Gene represented in genome annotation by CG4035, and previously located in the 67A8 or 67A2-B1 region by Lavoie et al. (1996) or Hernández et al. (1997), respectively.

sequence similarity in their carboxy-terminal moiety (Fig. 1a,b). *Drosophila* eIF4Es, except for eIF4E-8, contain the residues assumed to be involved in the recognition of the cap structure (Fig. 1a, depicted with lowercase s, r, p and m). However, the stretch of residues identified in human eIF4E to interact with the second nucleotide of m⁷GpppA (Fig. 1a, black horizontal line), that also contains the phosphorylatable serine (Fig. 1a, second asterisk) is partially conserved in *Drosophila* eIF4Es, and is totally absent in eIF4E-6. *Drosophila* eIF4E-3 also lacks the phosphorylatable serine. The lysine residue involved in the formation of a salt bridge with this serine is also absent in eIF4E-8 (Fig. 1a, first asterisk). Moreover, eIF4E-6 and eIF4E-8 show non-conservative substitutions in some of the residues proven to interact with eIF4G in other organisms (Fig. 1a, dots). The above sequence features found in *Drosophila* eIF4E-6 and eIF4E-8 suggest that these proteins might have an altered function. The identity values for the carboxy-terminal moiety of *Drosophila* eIF4E isoforms are shown in Fig. 1b. Interestingly, *Drosophila* eIF4E-8 shares more identity with human 4EHP (48%) and *C. elegans* IFE-4 (44%) than with other eIF4Es (approximately 30%). In these three proteins together with *Arabidopsis* nCBP, a tryptophan residue (corresponding to W56 of mouse eIF4E) involved in cap recognition is substituted by a tyrosine.

When the intron/exon organization of the eight *Drosophila* eIF4E genes was analyzed, it was noticed that genes *eIF4E-1,2*, *eIF4E-3*, *eIF4E-4* and *eIF4E-5*, which are clustered on region 65C–67A5 of chromosome 3L (Adams et al., 2000; Hernández et al., 1997; Lavoie et al., 1996) (Table 1), have two conserved exons of 100 and 88 bp or a fusion of them in the case of eIF4E-5 (Fig. 2). These exons encode the highly conserved carboxy-terminal moiety of the proteins. The position of the introns A, B and C (Fig. 2) interrupting these exons is also conserved. Thus, this cluster of genes may have originated by gene duplications from a common ancestor. Interestingly, introns B and D are conserved also in zebrafish eIF4E-1B (B. Joshi, personal communication), pepper, *A. thaliana*, rice, *S. pombe* and human *eIF4E-1* genes (Ruffel et al., 2004). By contrast, *eIF4E-6*, *eIF4E-7* and *eIF4E-8* do not share

any common exon/intron structure with the rest of *eIF4E* genes and they are scattered along the *Drosophila* genome (Fig. 2 and Table 1).

2.2. Functional properties of *Drosophila* eIF4Es

We tested the ability of the proteins synthesized in vitro to bind to the cap structure by affinity chromatography on m⁷GTP-sepharose (Fig. 3a). All *Drosophila* eIF4Es were able to bind to the cap. The translation of eIF4E-7 gave rise to several bands, probably due to the use of different methionines to initiate translation in our in vitro assay. All of them bound to the cap, in particular one at 37 KDa. The apparent lower cap-binding capacity of eIF4E-6 and eIF4E-8 might be explained because eIF4E-8 possesses three conservative (Y68, E102 and K164) and two non-conservative (Q124 and S169) substitutions in residues involved in cap recognition, while eIF4E-6 possesses a truncated carboxy-terminal part (Fig. 1a). Molecular modeling indicated that the cloud of positive charges formed by R157, K15 and K162 (for mouse eIF4E) surrounding the three phosphates of the cap is lost in *Drosophila* eIF4E-8, which only possesses one positive residue (K164) (not shown).

The ability of eIF4Es to bind *Drosophila* eIF4G (Hernández et al., 1998) and 4E-BP (Bernal and Kimbrell, 2000) was then investigated by using the yeast two-hybrid system (Bartel et al., 1993) (Fig. 3b). A strong interaction of eIF4G with eIF4E-1, eIF4E-2 and eIF4E-4 was observed, while an apparently weaker interaction of eIF4G with eIF4E-3, eIF4E-5 and eIF4E-7 was detected. We could not detect any interaction between eIF4G and eIF4E-6 or eIF4E-8. The same pattern of interactions was obtained with 4E-BP (Fig. 3b). The lack of interaction of eIF4E-6 and eIF4E-8 is in agreement with the existence of non-conservative substitutions in some of the residues shown to interact with eIF4G in other organisms (Fig. 1a, dots). Molecular modeling showed that P38 of mouse eIF4E which by hydrophobic interactions docks a pocket in the 4E-binding site of eIF4G is replaced by an arginine in *Drosophila* eIF4E-6 and eIF4E-8, losing in this way such contacts (not shown).

For a functional approach, we analyzed whether *Drosophila* eIF4E isoforms either alone or in combination with *Drosophila* eIF4G are able to complement the lack of eIF4E in a CDC33-knockout yeast strain (Altmann et al., 1989a). *Drosophila* eIF4E-1, eIF4E-3 and eIF4E-7 with the highest efficiency, and eIF4E-2 and eIF4E-4 with lower but significant efficiency, rescued the growth of the yeast mutant. In contrast, eIF4E-5, eIF4E-6, and eIF4E-8 were not able to support growth of the yeast mutant (Fig. 3c, upper panel). The failure of eIF4E-5, eIF4E-6, and eIF4E-8 to complement the yeast *eIF4E* mutant could be due to their inability to interact with yeast eIF4GI (TIF4631) as shown by two-hybrid system experiments (Fig. 3c, lower panel). It is possible that these *Drosophila* proteins are not able to

form a functional Dm-eIF4E/y-eIF4G complex in vivo. In an attempt to overcome this problem, we performed co-transfections of the yeast mutants to simultaneously express *Drosophila* eIF4E isoforms and eIF4G. The expression of *Drosophila* eIF4G did not modify the results obtained using endogenous yeast eIF4G (not shown). Finally, we transformed the yeast mutant strain CBY19, which carries a double knockout of *eIF4GI* and *eIF4GII* genes (Berset et al., 2003) with a plasmid carrying *Drosophila* eIF4G. Neither *Drosophila* eIF4G alone, nor in conjunction with any of the *Drosophila* eIF4Es complemented the lack of eIF4G in yeast, suggesting that the *Drosophila* eIF4E and eIF4G proteins fail to produce a functional complex for the yeast translational machinery. Altogether, the above results allow us to conclude that, at least, five *Drosophila* eIF4Es isoforms, namely eIF4E-1, eIF4E-2, eIF4E-3, eIF4E-4 and eIF4E-7 are functional in vivo.

2.3. Developmental expression of *Drosophila* eIF4E genes

We studied the expression of *eIF4E* genes throughout *Drosophila* development by Northern blot experiments using isoform-specific probes and total RNA. In contrast to eIF4E-1 and eIF4E-2 mRNAs, that were detected throughout the life cycle of *Drosophila* (Hernández and Sierra, 1995), single RNA transcripts of approximately 1.2, 1.2, 1.1 and 1.35 kb for eIF4E-3, eIF4E-4, eIF4E-5 and eIF4E-7, respectively, were found only from the third larva instar on. No transcript of eIF4E-6 was detected by this method. Two mRNAs (1.15 and 1.2 kb approximately) of eIF4E-8 were detected at early embryonic stage (Fig. 4a). This analysis was validated by quantitative, real-time RT-PCR using total RNA derived from different life stages and specific oligonucleotide primers for each mRNA except eIF4E-2 mRNA (Fig. 4b). eIF4E-1 polypeptide is encoded by two mRNAs (Hernández et al., 1997; Lavoie et al., 1996) and we used primers that recognize the two transcripts together. The mRNAs of eIF4E-1 were detected during all the developmental stages studied and at a higher level than the RNAs encoded by the other genes, particularly in early embryo

stage. eIF4E-8 transcript was also detected in all stages but at a much lower level. We found eIF4E-3 transcript at very low levels from late embryos that increased significantly from the third instar larvae. The transcripts of eIF4E-4, eIF4E-5 in particular, and of eIF4E-6 and eIF4E-7 at much lower levels, were detected from the third instar larvae onwards (Fig. 4b). In situ hybridization of eIF4E-3 and eIF4E-8 in embryos detected a very weak and ubiquitous signal in both cases (data not shown).

2.4. Cap-dependent translation relies on eIF4E-1 in embryos

The above results support the notion that only the transcripts of eIF4E-1, eIF4E-3 and eIF4E-8 are present in *Drosophila* embryos. To examine whether the corresponding proteins were present in an active eIF4F complex, we isolated such a complex from embryos 0–18 h-old by affinity chromatography on a m⁷GTP-Sepharose column (Zapata et al., 1994), analyzed it by SDS/PAGE and the protein bands were identified by MALDI-TOF mass spectroscopy. As already described (Zapata et al., 1994) *Drosophila* eIF4F consists of eIF4E-1 and eIF4G polypeptides, although traces of additional polypeptides, namely HSP70, PABP and a putative RNA-binding protein encoded by the gene CG2950 were also detected (Fig. 5a). When the amount of eIF4F analyzed was scaled up 30 fold, traces of additional polypeptides were detected and further identified by LC-MSMS (see Fig. 5b). However, only eIF4E-1 and a trace amount of eIF4E-8 were found. In *Drosophila* only one eIF4G protein has been characterized (Hernández et al., 1998; Zapata et al., 1994), which is present in the eIF4F complex from embryos (Fig. 5a,b). Lately another two putative *eIF4G* genes (CG10192 and CG3845) were identified in the *Drosophila* genome (Adams et al., 2000), but none of them was detected in the embryos eIF4F. The above results suggest that the bulk of cap-recognition activity in embryos relies on eIF4E-1.

To confirm this hypothesis we analyzed the lack of eIF4E-1 function in embryos in the context of the whole

Fig. 1. Sequence comparison of *Drosophila* eIF4E isoforms. (a) Alignment of deduced amino acid sequences of *Drosophila* eIF4E isoforms with those from mouse (m4E) (Jaramillo et al., 1991) and yeast (y4E) (Altmann et al., 1987) counterparts. The accession numbers used are: *Drosophila* eIF4E-1 (U16139) (Hernández and Sierra, 1995), eIF4E-2 (U63033) (Hernández et al., 1997; Lavoie et al., 1996), eIF4E-3 (CG8023), eIF4E-4 (CG10124), eIF4E-5 (CG8277), eIF4E-6 (CG1442), eIF4E-7 (CG32859) (Birney, 2004; Lasko, 2000) and eIF4E-8 (CG33100) (Birney, 2004); Mouse eIF4E (M61731); and yeast eIF4E (M15436). Sequences of *Drosophila* eIF4Es were corroborated by PCR-amplification from cDNA libraries and sequencing. Identical residues in at least seven proteins are in black boxes. Identical residues in four, five or six proteins, or conservative substitutions in at least five proteins are depicted in grey boxes. Mutations among the following groups of amino acids were considered conservatives: P; G and A; S and T; K, R and H; E, D, Q, and N; I, L, M, V, C, Y, F and W. Carboxy-terminal moiety of proteins is indicated by an arrowhead. Residues involved in the recognition of the cap (Gross et al., 2003; Marcotrigiano et al., 1997; Matsuo et al., 1997; Tomoo et al., 2002, 2003) are pointed with a lowercase as follows: s, Trp binding the guanine by π - π interactions; g, Glu recognizing the guanine ring; r, Asp stabilizing Arg157 (numbers refers to mouse eIF4E), which interacts with the phosphate groups; p, additional residues interacting with the phosphate groups; m, Trp recognizing the cap methyl group; asterisks point the serine residue which is phosphorylated in mammals and *Drosophila* eIF4E-1 (Flynn and Proud, 1995; Joshi et al., 1995; Lachance et al., 2002; Pyronnet et al., 1999; Waskiewicz et al., 1999) and the lysine residue forming a salt bridge to stabilize the interaction with the cap (Marcotrigiano et al., 1997). Residues identified in mammalian or yeast eIF4E to interact with eIF4G and 4E-BP (Gross et al., 2003; Marcotrigiano et al., 1997, 1999; Matsuo et al., 1997; Ptushkina et al., 1999; Tomoo et al., 2002, 2003) are labeled with dots. The stretch of residues 204–211 on human eIF4E identified to interact with the second nucleotide of m⁷GpppA (Tomoo et al., 2002, 2003) is pointed with a horizontal black line. Gly113, Gly179 and Glu73 implicated in the activity of yeast eIF4E (Altmann and Trachsel, 1989; Brenner et al., 1988) are labeled with a cross. (b) Identity values among the carboxy-terminal moiety of *Drosophila* eIF4Es. Identity values among amino-terminal parts of the proteins are below 20%.

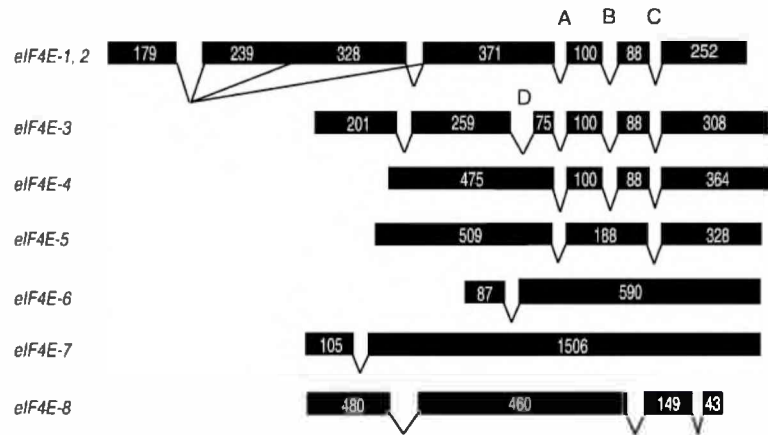


Fig. 2. Structure of the *Drosophila* eIF4E genes. The size of exons (black boxes) for *eIF4E-1,2* gene was adopted from (Hernández et al., 1997; Lavoie et al., 1996), and for the rest of eIF4E genes from the Ensembl Project of the Sanger Institute (Birney, 2004). Numbers refer to the size in nucleotides. Lines, and capitals A, B, C and D represent introns.

organism. We used the six non-identified lethal alleles obtained by Leicht and Bonner (Leicht and Bonner, 1988) in the region 67A2-B1 of the third chromosome, where this gene was mapped (Hernández et al., 1997). One of these alleles, namely *l(3)67Af*, was lately identified to be an embryonic null mutant of the *eIF4E-1,2* gene (Hernández et al., 2004b; Lachance et al., 2002). We then investigated whether these mutants could be rescued by the expression of the transgene *UAS-eIF4E1* under the control of the *T80-Gal4* driver, which drives the expression of the transgene from stage 11 onwards in a weak but ubiquitous way (Hrdlicka et al., 2002). We performed the phenotypic rescue experiments of the mutants in transheterozygosis with the deficiency *Df(3L)29A6(66F3;67B1)*, *ri¹ p^p* (Leicht and Bonner, 1988) in order to avoid possible lethality due to the homozygosis of other mutations in essential genes in each mutant not detected previously. In this way, the lethality by homozygosis is in each case unambiguously attributed to the mutant analyzed. The rescue was determined as the number of individuals that reached pupae or adulthood. As shown in Table 2, the expression of the transgenic *UAS-eIF4E1* has the ability to rescue the *l(3)67Af* mutant until adulthood. None of the other five alleles in this region were rescued, meaning a specific complementation of the eIF4E function by the *UAS-eIF4E1* activity. These results support an essential role for eIF4E-1 in embryos that is not redundant with the activity of other eIF4E genes found in *Drosophila*, in particular with genes whose expression was detected during embryonic stages, namely eIF4E-3 and eIF4-8.

2.5. Overexpression of *eIF4E-1* causes phenotypic defect in a dose-dependent manner

We also studied the effect of overexpression of eIF4E-1 in embryos using the transgenic flies *UAS-eIF4E1* and different Gal4 drivers. No phenotypic effect was obtained with

the *T80-Gal4*, which drives the expression of the transgene from stage 11 onwards (Hrdlicka et al., 2002). We then used the *NGT40-Gal4* (Li and Gergen, 1999) to overexpress *UAS-eIF4E1* from the moment of oocyte fecundation. When we allowed the first 8 h of development to occur at 17 °C, and then either switched the embryos to 29 °C or permitted to continue at 17 °C (control), no mortality was detected and adults had no phenotype defects. When the embryos were allowed to grow throughout at 29 °C to overexpress the *UAS-eIF4E1* transgene from the beginning of development, we obtained 20% embryo mortality. Cuticle preparation of first instar larva *NGT40-Gal4/UAS-eIF4E1* showed defects in segmentation (Fig. 6a). A more dramatic effect was observed when *UAS-eIF4E1* was overexpressed early in development by using the *en-Gal4* driver. The growth of the flies at 17 °C during the complete life cycle (control) produced normal adults. When the eggs were allowed to grow at 25 or 29 °C from the moment of fertilization, all animals died in early syncytial blastoderm stage. When the eggs *en-Gal4/UAS-eIF4E1* were grown for the first 72 h at 17 °C and then switched to 25 or 29 °C, all eggs reached adulthood with no phenotypic defects. As shown in Fig. 4b, the highest amount of eIF4E-1 mRNA was found in 0–3 h-old embryos. We then performed Western blot experiments to analyze the relative amounts of eIF4E-1 protein throughout the embryogenesis of *Drosophila*. In agreement with mRNA amounts observed in Fig. 4b, the highest abundance of eIF4E-1 protein is detected in embryos 0–3 h-old and decreases as embryogenesis proceeds (Fig. 6b). A likely explanation for the observed phenotype upon overexpression of eIF4E-1 only in early embryonic stages with the *en-Gal4* is that there is a threshold in the amount of eIF4E-1 to be surpassed in order to produce phenotypic effects. We then analyzed the overexpression of eIF4E-1 in a later stage of development. By using *GMR-Gal4* (Freeman, 1996), *sev-Gal4* (Reiter et al., 1996) and *ey-Gal4* (Halder et al., 1995) drivers, we overexpressed the *UAS-eIF4E1*

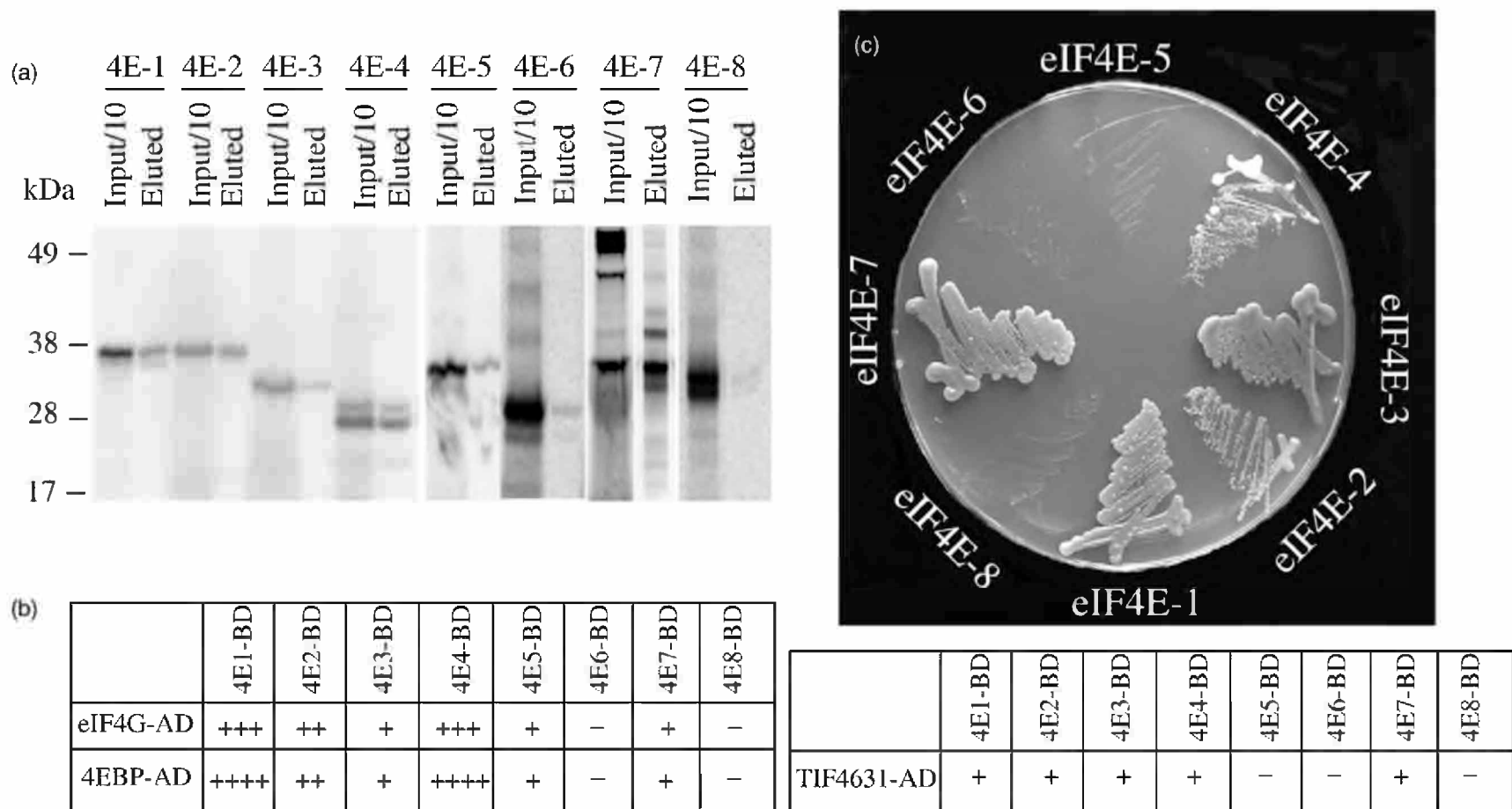


Fig. 3. Functionality of *eIF4E* genes. (a) Binding to cap of eIF4E isoforms. Autoradiography of [35 S]-labeled proteins subjected to cap-binding analysis by affinity chromatography on a m⁷GTP-Sepharose column. Specific cap-bound proteins were eluted with buffer containing 100 μ M m⁷GTP and further resolved by SDS-PAGE (12.5% acrylamide), detected and quantified with the help of a phosphorimager. For each case, one-tenth of the analyzed input was loaded on the gel. Molecular mass markers are indicated on the left. (b) Interaction of *Drosophila* eIF4E isoforms with *Drosophila* eIF4G and 4E-BP observed when using the yeast two-hybrid. Bait eIF4E (4E) isoforms were fused to the Gal4-binding domain (BD). Prey eIF4G and 4EBP were fused to the Gal4-activator domain (AD). The relative strength of the interactions is indicated. c, upper) Phenotypic rescue of a null yeast *eIF4E* mutant by *Drosophila* eIF4E isoforms. The *S. cerevisiae* strain T93C (Altmann et al., 1989b), a conditionally lethal allele of yeast *CDC33* (eIF4E) gene, was transformed with DNA constructs expressing different *Drosophila* eIF4E isoforms and tested for complementation of endogenous eIF4E. Transformed yeast cells were streaked on YPGal (2% galactose)-agar plates and colony growth was analyzed at 30 °C. eIF4E-1, eIF4E-2, eIF4E-3, eIF4E-4 and eIF4E-7 supported growth of the mutant yeast. eIF4E-5, eIF4E-6 and eIF4E-8 did not support growth. Same results were obtained when the yeast mutant was cotransformed with eIF4E constructs in conjunction with *Drosophila* eIF4G (not shown). c, lower) Interaction of *Drosophila* eIF4E isoforms fused to the Gal4-binding domain (BD) with yeast TIF4631 (eIF4GI) fused to the Gal4-activator domain (AD) as studied by the yeast two-hybrid system. The relative strength of the interactions is shown.

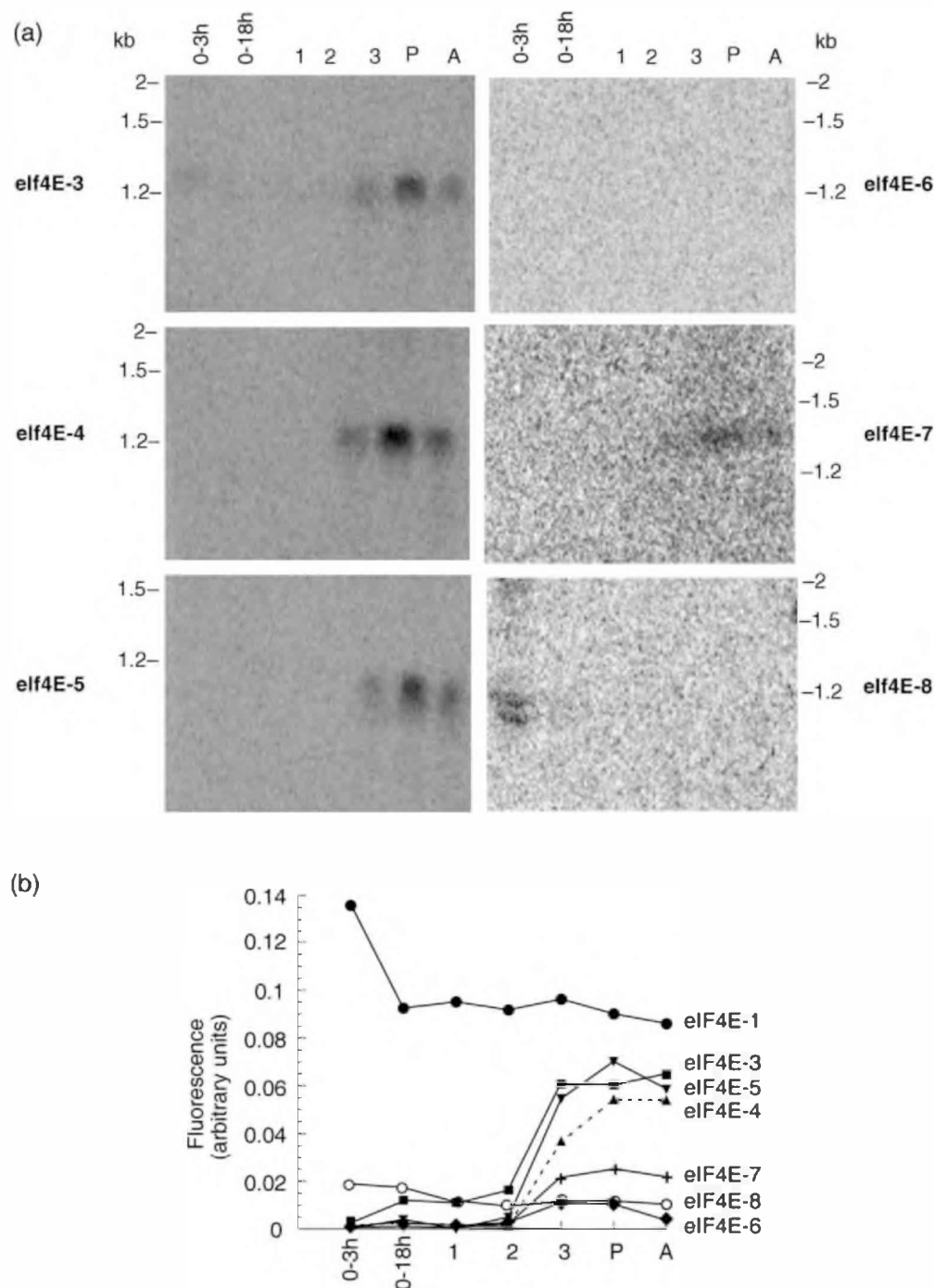


Fig. 4. Expression of *eIF4E* genes during *Drosophila* life cycle. (a) Northern blot detection of *eIF4E-3*, *eIF4E-4*, *eIF4E-5*, *eIF4E-6*, *eIF4E-7* and *eIF4E-8* mRNAs using isoform specific [32 P]-labeled probes on total RNA derived from early embryos (0–3 h), embryos (0–18 h), first (1), second (2) and third (3) instar larvae, pupae (P) and adults (A). The blots for *eIF4E-6*, *eIF4E-7* and *eIF4E-8* were exposed 5 times longer than the rest. (b) Relative levels of *eIF4E* isoform mRNAs measured by quantitative real time RT-PCR of total RNA from the same stages as displayed in (a). Data represent the average of three independent experiments with a variance of 5%.

transgene in and behind the morphogenetic furrow, in a subset of photoreceptors and the cone cells, or in the precursors of ommatidia, respectively, of the developing eye imaginal disc. In all cases, analysis of the adult eye showed no phenotypic effect when the transgene *UAS-eIF4E1* was present in a single copy (not shown).

When the transgene was present in two copies, cell proliferation, abnormal growth, severe disturbance of the periodic pattern of ommatidia lattice and the presence of extra chaete were observed (Fig. 6c). These results support our hypothesis that a threshold of *eIF4E-1* level has to be surpassed in order to produce phenotypic effects in the fly.

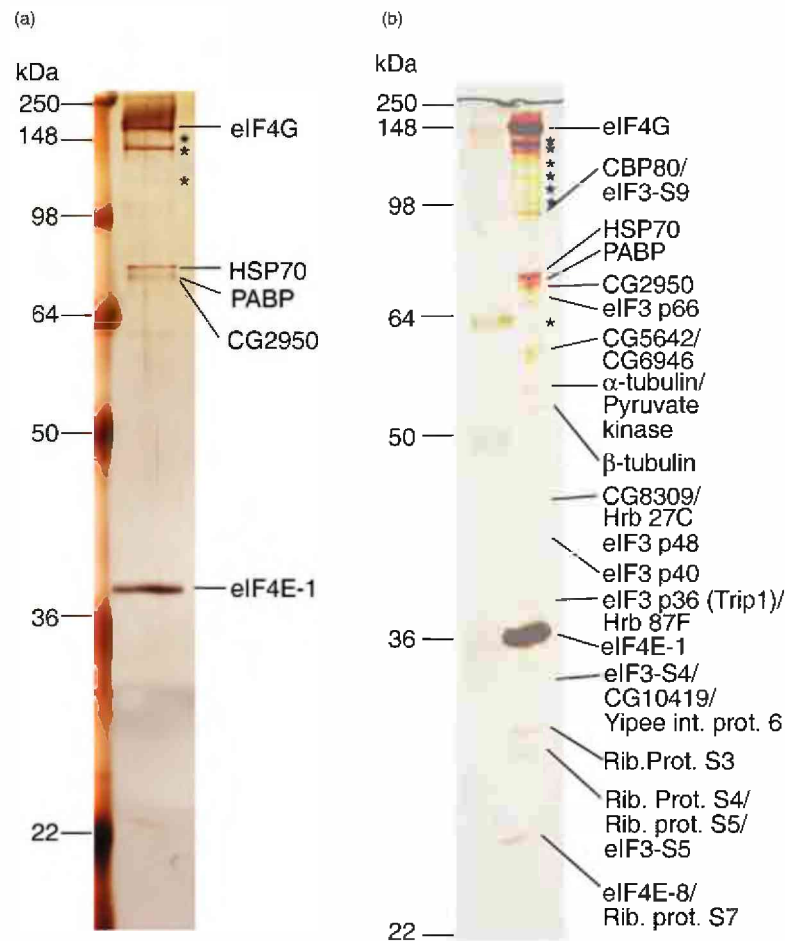


Fig. 5. Proteomics of *Drosophila* eIF4F complex from embryos 0–18 h-old. eIF4F complex was purified by affinity chromatography on a m⁷GTP-Sepharose column and resolved by SDS-PAGE (Zapata et al., 1994). (a) Silver staining of 600 ng of eIF4F complex. The bands were analyzed by mass spectroscopy using the thin-layer method and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF). (b) Silver staining of 18 µg of eIF4F resolved by SDS-PAGE. The bands were analyzed by liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS). The accession numbers of the identified proteins are: eIF4G (CG10811); CBP (CG7035); eIF3-S9 (CG4878); HSP70 (CG4264); PABP (gi|418855); RNA binding protein (KH domain) (CG2950); eIF3 p66 (gi|7141239); CG5642; RNA splicing protein (CG6946); alpha-tubulin (CG1913, CG2512); Pyruvate kinase (gi|3108349); beta-tubulin (CG9277); metal dependent phosphohydrolase (HD domain) (CG8309); Hrb27 (involved in RNA localization and RNA import (gi|17136728); eIF3 p48 (CG9677); eIF3 p40 (CG9124); eIF3 p36 (CG8882); hnRNP Hrb87F (involved in RNA splicing and RNA localization) (gi|8318); eIF4E-1 (CG4035); eIF3 p44 (CG8636); RNA splicing protein (CG10419); Yipee interacting protein (gi|6752674); Ribosomal protein S3 (CG6779); Ribosomal protein S5 (CG5920); Ribosomal protein S4 (CG11276); eIF3 p47 (CG9769); eIF4E-8 (CG33100); Ribosomal protein S7 (CG7014). Asterisks point to degradation products of eIF4G.

As assessed by Western blot, at least four times more eIF4E-1 protein on eyes in transgenic flies is required to produce a phenotypic effect (Fig. 6d).

3. Discussion

3.1. Functional diversity of *Drosophila* eIF4E isoforms

The ability of the eight *Drosophila* eIF4E isoforms to bind to cap, to eIF4G and to 4E-BP, and to support cell growth in a yeast mutant deficient for eIF4E, demonstrate that eIF4E-1, eIF4E-2, eIF4E-3, eIF4E-4 and eIF4E-7 support translation initiation and suggest that they may be functional equivalents

Table 2

Phenotypic rescue of mutant *l(3)67Af* by over-expression of transgenic eIF4E1

Genotype	Temperature (°C) ^a	Pupae ^b	Adults ^b
<i>l(3)67Af/Df(3L)29A6</i>	25	0.64% (1/156)	0%
<i>GAL4-T80/UAS-4E1; l(3)67Af/Df(3L)29A6</i> (3/19)	25	16% (3/19)	0%
<i>GAL4-T80/UAS-4E1; l(3)67Af/Df(3L)29A6</i>	29	8.3% (5/60)	6.7% (4/59)

^a At 18 °C no rescue to pupa or adult stages was obtained.

^b The number of individuals of the corresponding genotype obtained respect the total progeny is indicated in brackets. 33% would be expected for total rescue.

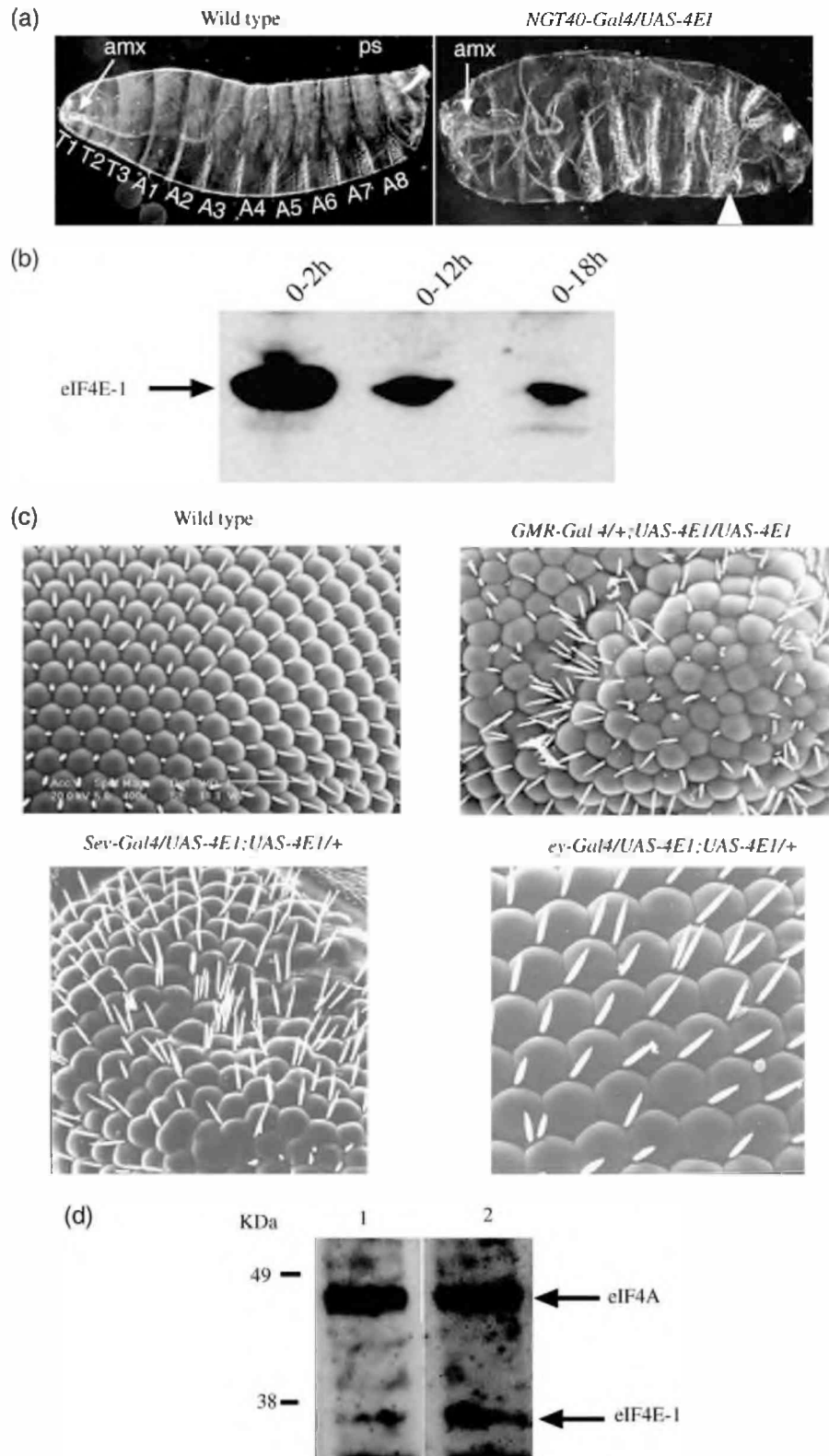


Fig. 6. Effect of overexpression of eIF4E-1 in embryo and eyes. Cuticles of wild type and *UAS-eIF4E1/NGT40-Gal4* first instar larvae. Thoracic (T1–T3) and abdominal (A1–A8) segments, as well as the antenno-maxillary complex (amx) and posterior spiracles (ps) are pointed. Fusions of segments are pointed by a triangle. (b) Analysis of eIF4E-1 in embryos 0–2, 0–12 and 0–18 h-old by Western blot. 5 μ g of total protein extracts of embryos was loaded per lane, resolved by SDS-PAGE, blotted and analyzed with an anti-eIF4E-1 antibody (Maroto and Sierra, 1989). (c) Scanning electronic microscopy of adult eyes surfaces from wild type flies (400 \times) or flies overexpressing two copies of the *UAS-eIF4E1* transgene on the developing eye under the Gal4 drivers *Glass* (*GMR*) (400 \times), *sevenless* (*sev*) (400 \times) or *eyeless* (*ey*) (500 \times). (d) Western blot to detect eIF4E-1 and eIF4A (control) in total extracts of five heads of *white* (1) or flies carrying two copies of transgenic eIF4E-1 (2).

in vivo. On contrary, eIF4E-6 and eIF4E-8 do recognize cap with lower efficiency than other eIF4Es, and did not interact with eIF4G nor were able to support cell growth in the yeast mutant. They are the unique isoforms carrying non-conservative amino acid substitutions (two in the case of eIF4E-6 and six in the case of eIF4E-8) at important residues for the interaction of eIF4E with eIF4G. In conclusion, eIF4E-6 and eIF4E-8 may be either negative regulators of translation or simply non-functional proteins. On the other hand, serine 251 of eIF4E-1 is critical for the function of eIF4E-1 in *Drosophila* (Lachance et al., 2002), but eIF4E-3 that lacks this serine is able to support cell growth in the yeast eIF4E-mutant. Since yeast eIF4E also lacks this serine we suppose there is no requirement for it in the function of eIF4E from this organism.

In spite of the existence of eight isoforms for eIF4E in *Drosophila*, here we have shown that in *Drosophila* embryos the cap-dependent translation relies mainly on eIF4E-1 and that the activity of this factor is essential throughout embryogenesis. This has important implications for development. During oogenesis, the repression of *oskar* mRNA in the posterior pole of the oocyte is essential for germ line formation and patterning (Johnstone and Lasko, 2001). During early embryogenesis, repression of *caudal* mRNA expression in the anterior part of the embryo is required for appropriate assembly of the head segments (Niessing et al., 1999). Both maternal mRNAs are regulated by the interaction of their repressors CUP and BICOID with eIF4E-1 (Nakamura et al., 2004; Niessing et al., 1999, 2002; Wilhelm et al., 2004). Here we showed that the likely isoform to be involved in translation of early mRNAs is eIF4E-1. The analysis of the Me31B complex from ovary extracts presented by Nakamura et al. (2004) showing that only eIF4E-1 is enriched in the *oskar* mRNA repression complex also supports the idea of a key role for eIF4E-1 in early embryogenesis.

3.2. Chimeric translational machineries

In spite of the evolutionary conservation of the translational machinery across the eukaryotic phyla, only some eIF4E isoforms can complement for the lack of the yeast eIF4E. They include human eIF4E-1 (Altmann et al., 1989a), *A. thaliana* eIF4E (Rodríguez et al., 1998), zebrafish eIF4E-1A (Robalino et al., 2004), and five *Drosophila* eIF4E isoforms (this study). In contrast, other initiation factors involved in mRNA recruitment and scanning do not allow for cross-complementation: neither mouse eIF4A (Prat et al., 1990) nor *Drosophila* factors eIF4A, eIF4B (Hernández et al., 2004c) or eIF4G (this study) substitute for their yeast counterparts. This suggests that the pathways regulating eIF4E activity may be universally more conserved than those for other factors. It will be worthwhile to test whether other initiation factors (e.g. eIF3, eIF2, etc.) are able to substitute for their homologs, and whether eIF4E

is interchangeable between phyla other than yeast (e.g. between plants and mammals).

3.3. Is eIF4E a limiting factor during initiation of translation?

Increased eIF4F formation is closely related to enhanced protein synthesis and thus to cell growth. eIF4E is referred to as the limiting factor in the formation of eIF4F complex (Sonenberg and Gingras, 1998) because it is less abundant in some mammalian cells than eIF4G (Duncan et al., 1987; Hiremath et al., 1985). However, in most cases, overexpression of eIF4E in mammal cells leads to malignant transformation and non-controlled cell proliferation only when overexpressed in cell cycle-sensitized cells or when co-expressed together with other proto-oncogenes (de Benedetti and Graff, 2004; Dua et al., 2001; Rosenwald, 2004; Sonenberg and Gingras, 1998). In *S. cerevisiae*, even 100 fold overexpression of eIF4E had only a minor effect on growth rates (Lang et al., 1994). In *Xenopus* (Wakiyama et al., 2001) and *S. pombe* (Hashemzadeh-Bonehi et al., 2003), overexpression of either of the two eIF4Es modestly increases translation in oocytes or had not affected cell growth, respectively. Here we observed that the overexpression of *eIF4E-1* transgene produces phenotypic defects in early embryos (a time when endogenous eIF4E-1 is expressed most strongly) or when it was expressed in more than one copy in the developing eye. As we have recently demonstrated for *Drosophila* eIF4B (Hernández et al., 2004c), here we provide in vivo evidence for phenotypic defects produced by changes in eIF4E levels in a non-oncogenic or cell cycle-sensitized genetic background, both in embryo and in the developing eye. These defects are produced in a dose-dependent manner. Altogether, these data suggest that in a wild type genetic background only a very high level of overexpression of eIF4E-1 might result in phenotypic effects. This would explain why no phenotypic defects were obtained by overexpression of *Drosophila* eIF4E-1 in previous studies (Lachance et al., 2002; Zhang et al., 2000). It seems likely that those cells having an excess of eIF4E over eIF4G, like in rabbit reticulocyte lysates (Rau et al., 1996), yeast (von der Haar and McCarthy, 2002) and *Drosophila* embryos (Hernández and Rivera-Pomar, unpublished), are less sensitive to a further increase in the amount of free eIF4E. This implies that eIF4G, not eIF4E, is the limiting factor in the formation of eIF4F during the initiation of translation.

4. Experimental procedures

4.1. Identification and sequence analysis of eIF4E isoforms

Drosophila eIF4E-1 sequence was taken from Hernández and Sierra (1995) and that for eIF4E-2 from Hernández et al. (1997). Sequences for *Drosophila* annotated genes CG8023

(*eIF4E-3*), CG10124 (*eIF4E-4*), CG8277 (*eIF4E-5*), CG1442 (*eIF4E-6*), CG32859 (*eIF4E-7*) and CG33100 (*eIF4E-8*) were taken from Lasko (2000) and from the Ensembl Project (Birney, 2004), program version number 21.3a.1, from the Wellcome Trust Sanger Institute (Cambridge, UK; web site: http://www.ensembl.org/Drosophila_melanogaster). Sequences lineup were performed using the CLUSTAL W algorithm (Thompson et al., 1994) in the Megaline program of the DNA Star software package and optimized by eye. Sequence identity values were obtained using the programs BESTFIT and GAP, of the *Genetics Computer Group Sequence Analysis Software*, Wisconsin, USA.

4.2. Plasmids construction

The open reading frames (ORF) of all eight *eIF4E* were PCR-amplified using sequence-specific primers (Metabion). For *eIF4E-1*, *eIF4E-3* and *eIF4E-4*, pBS-*eIF4E-1* (Hernández and Sierra, 1995) and ESTs LD034967 and GH01027 from the Berkeley *Drosophila* Genome Project were used as templates, respectively. *eIF4E-2*, *eIF4E-5*, *eIF4E-6*, *eIF4E-7* and *eIF4E-8* were amplified from a cDNA library constructed from pupae poly A⁺-RNA. After PCR amplification, cDNA fragments were cloned onto pCR2.1 vector (Invitrogen) and both strands fully sequenced, thus creating the constructs 4Es-pCR2.1. All *Drosophila eIF4Es* and *Drosophila eIF4G* (Hernández et al., 1998) ORFs were further subcloned onto the vectors p301-TRP1/GAL1 and p301-HIS3/GAL1, which allow for expression of cDNAs upon induction on galactose-containing media (Altmann et al., 1993), to create the respective plasmids 4Es-TRP1, 4Es-HIS3 or 4G-TRP1. Full-length *eIF4E-1* cDNA (Hernández and Sierra, 1995) was subcloned onto the vector pUAST (Brand and Perrimon, 1993) to create the plasmid pUAS-4E1. *Drosophila eIF4Es*, *eIF4G* and 4E-BP (Bernal and Kimbrell, 2000) ORFs were cloned onto the vectors pGBT9 or pGAD424 (Clontech), respectively, to build the bait constructs 4Es-BD (Binding Domain) or the prey construct 4EBP-AD (Activator Domain), respectively.

4.3. Northern blot and quantitative real-time RT-PCR

Total RNA of staged wild type *Drosophila* (Oregon R) was isolated using the RNeasy Mini Kit (Qiagen), digested with RNase-free DNase I (Qiagen) and quantified by spectrophotometry. Northern Blot was performed as described (Hernández and Sierra, 1995) using 100 ng of [³²P]-labelled DNA probes and hybridized at 50 °C. 100 ng of total RNA per reaction was used as template for quantitative real-time RT-PCR using the QuantiTect SYBR green RT-PCR kit (Qiagen) in an Engine Opticon System (M.J. Research Inc.). Primers were sequence-specific 25-mer oligonucleotides (Metabion) designed to amplify 100 bp-long fragments in each case.

4.4. Proteins labeling and cap binding analysis

Two microgram of each pCR2.1-*eIF4E* DNA was subjected to transcription/translation in vitro using the TNT-coupled Reticulocyte Lysate System (Promega) in the presence of a [³⁵S]Met and [³⁵S]Cys mixture (14.3 mCi/ml; Amersham), as described by the manufacturer. Labeled proteins were subjected to cap-binding analysis by affinity chromatography on a m⁷GTP-Sepharose column (Pharmacia) according to (Maroto and Sierra, 1989) as follows. 150 µl of TNT reactions containing the labeled proteins were dialyzed against buffer A (20 mM HEPES pH 7.6, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride). Eight-hundred and fifty microliter of buffer A were added and the total volume (1 ml) was mixed with 10 µl of m⁷GTP-Sepharose resin (Pharmacia, Inc.) and gently shaken for 4 h. The resin was collected by centrifugation (370×g for 5 min), washed twice with 1 ml buffer A, poured into a column and further extensively washed with 10 ml buffer A. Nonspecifically bound proteins were eluted by washing the column with 10 volumes of buffer A containing 0.1 mM GTP. Specific cap-bound proteins were eluted with buffer A containing 100 µM m⁷GTP, resolved by SDS-PAGE (12.5% acrylamide) and quantified with the help of a phosphorimager. All steps were performed at 4 °C.

4.5. Western blot

Western blotting was performed using anti-*eIF4E-1* (1:1000) (Maroto and Sierra, 1989) or anti-*eIF4A* (1:5000) (Hernández et al., 2004a) polyclonal antibodies, HRP-coupled goat anti-rabbit secondary antibody (1:20,000) (Dianova), and the ECL detection kit (Amersham Pharmacia Biotech).

4.6. Mass spectroscopy

Proteins were precipitated with 3 volumes ethanol and 0.1 volumes sodium acetate pH 5.2 for 2 h at −20 °C, centrifuged at 14,000 rpm for 30 min at 4 °C, washed with ethanol 80% and further air-dried. Proteins were resolved by SDS-PAGE and visualized by silver staining. Mass spectrometry of the proteins was performed by the thin-layer method and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) as described (Hartmuth et al., 2002) in a Bruker Reflex IV apparatus. Recorded spectra were analyzed using the ToF 5.1.1 software (Bruker, Bremen, Germany) and the proteins identified in the Nonredundant Database from the National Center for Biotechnology Information by using MASCOT (Matrix Science, London) as a searching tool. The less-abundant proteins that could not be identified by MALDI-TOF were identified by liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) as described (Hartmuth et al., 2002) in an Ultimate HPLC system containing a Switchos II

pre-column switching device and a Famos autosampler (LC Packings, Idstein, Germany) coupled to an orthogonal quadrupole time-of-flight mass spectrometer (Q-ToF 1, Micromass, Manchester, UK). The electrospray was generated with fused-silica 10- μ m PicoTip needles (New Objectives, Cambridge, MA), which were operated at 2.8–3.5 kV.

4.7. Yeast two-hybrid assays

Saccharomyces cerevisiae strain Y190 was co-transformed with bait and prey constructs and plated on selective medium (-trp, -leu). The colonies were then transferred to selective medium (-trp, -leu, -his) containing 25 mM 3-amino-1,2,4-triazole (Sigma), and the positive interacting colonies assayed for b-galactosidase activity on paper filter as described (Bartel et al., 1993).

4.8. In vivo complementation in *S. cerevisiae*

4Es-TRP1, 4Es-HIS3 or 4G-TRP1 constructs were used to transform the *S. cerevisiae* strains T93C that carries a conditionally lethal allele of yeast *CDC33* (eIF4E) gene (Altmann et al., 1989b), and CBY19 that carries a double knockout of *TIF4631* (eIF4GI) and *TIF4632* (eIF4GII) genes (Berset et al., 2003). After transformation, yeast cells were replica plated on YPGal (2% galactose) medium and tested at 30 °C for their capacity to complement the lack of endogenous eIF4E or eIF4G as described (Altmann et al., 1989a).

4.9. Fly stocks, transgenic flies preparations and Scanning electron microscopy of eyes

Flies strains were raised as described (Ashburner, 1989). Construct *UAS-eIF4E1* was used to generate transgenic flies *yw*; *P{w UAS-eIF4E1}* as described in (Rubin and Spradling, 1982) by microinjection in *yw* embryos. Ectopic overexpression of transgene *UAS-eIF4E1* was achieved by crossing flies *yw*; *P{w UAS-eIF4E1}* with the early embryonic Gal4 drivers *en-Gal4*, *NGT40-Gal4*, the late embryonic driver *T80-Gal4*, or the eye imaginal disc drivers *Gal4 sev-Gal4*, *ey-Gal4* and *GMR-Gal4*. Cuticle preparations were done as described in Ashburner (1989). For Scanning electron microscopy (SEM), adult flies were subjected to sequential ethanol dehydration, critical point drying and then coated with a gold-palladium mix in a Cool Sputter Coater (Fisons Instruments, UK). Images were acquired using a Digital Scanning Microscope DSM960 (Zeiss, Germany).

4.10. Rescue of a null mutant for eIF4E

The mutant *l(3)67A^f ri¹ e⁴/TM3, Sb¹* (Leicht and Bonner, 1988), which is a null mutant for the gene *eIF4E-1,2* (Hernández et al., 2004b; Lachance et al., 2002), and

the deficiency mutant *Df(3L)29A6(66F3;67B1), ri¹ p⁺/TM3* (Leicht and Bonner, 1988), were obtained from Bloomington Stock Center (USA). Stocks *Gal4-T80/SM6a-TM6b/l(3)67A^f* were generated and crossed to *UAS-eIF4E1/SM6a-TM6b/Df(3L)29A6*. Pupae and adults *w*; *Gal4-T80/UAS-eIF4E1; l(3)67A^f/Df(3L)29A6* were identified by their lack of the *Cy* and *Tb* markers.

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